

COMPOUND

Related Applications

This application is a continuation-in-part of allowed U.S. application Serial No. 09/319,213, filed November 23, 1999, which is a continuation-in-part of allowed U.S. application Serial No. 09/111,927, filed July 8, 1998, now U.S. Patent No. 6,011,024, and a continuation-in-part of PCT/GB97/03352, filed December 4, 1997, designating the U.S. and published as WO 98/24802, with a claim of priority from Great Britain application no. 9625334.9, filed December 5, 1996. This application is also a continuation-in-part of allowed U.S. application Serial No. 09/724,986, filed November 28, 2000, which is a continuation-in-part of PCT/GB99/01835, filed June 10, 1999, designating the U.S. and published as WO 99/64013, with claims of priority from Great Britain application nos. 9812535.4, filed June 10, 1998 and 9910167.7 filed April 30, 1999. All of the foregoing applications, as well as all documents cited in the foregoing applications ("application documents") and all documents cited or referenced in application documents are hereby incorporated herein by reference. Also, all documents cited in this application ("herein cited documents") and all documents cited or referenced in herein cited documents are hereby incorporated herein by reference.

The present invention relates to a compound.

5 In particular the present invention relates to a compound and to a pharmaceutical composition comprising the compound.

Evidence suggests that oestrogens are the major mitogens involved in promoting the growth of tumours in endocrine-dependent tissues, such as the breast and endometrium.

10 Although plasma oestrogen concentrations are similar in women with or without breast cancer, breast tumour oestrone and oestradiol levels are significantly higher than in normal breast tissue or blood. *In situ* synthesis of oestrogen is thought to make an important contribution to the high levels of oestrogens in tumours and therefore specific inhibitors of oestrogen biosynthesis are of potential value for the treatment of endocrine-dependent
15 tumours.

Over the past two decades, there has been considerable interest in the development of inhibitors of the aromatase pathway which converts the androgen precursor androstenedione to oestrone. However, there is now evidence that the oestrone sulphatase
20 (E1-STS) pathway, i.e. the hydrolysis of oestrone sulphate to oestrone (E1S to E1), as opposed to the aromatase pathway, is the major source of oestrogen in breast tumours^{1,2}. This theory is supported by a modest reduction of plasma oestrogen concentration in postmenopausal women with breast cancer treated by aromatase inhibitors, such as aminoglutethimide and 4-hydroxyandrostenedione^{3,4} and also by the fact that plasma E1S
25 concentration in these aromatase inhibitor-treated patients remains relatively high. The long half-life of E1S in blood (10-12 h) compared with the unconjugated oestrogens (20 min)⁵ and high levels of steroid sulphatase activity in liver and, normal and malignant breast tissues, also lend support to this theory⁶.

PCT/GB92/01587 teaches novel steroid sulphatase inhibitors and pharmaceutical compositions containing them for use in the treatment of oestrone dependent tumours, especially breast cancer. These steroid sulphatase inhibitors are sulphamate esters, such as N,N-dimethyl oestrone-3-sulphamate and, preferably, oestrone-3-sulphamate (otherwise known as "EMATE").

Some of the compounds disclosed in PCT/GB92/01587 are shown in Figure 1.

It is known that EMATE is a potent E1-STS inhibitor as it displays more than 99% inhibition of E1-STS activity in intact MCF-7 cells at 0.1 mM. EMATE also inhibits the E1-STS enzyme in a time- and concentration-dependent manner, indicating that it acts as an active site-directed inactivator^{7,8}. Although EMATE was originally designed for the inhibition of E1-STS, it also inhibits dehydroepiandrosterone sulphatase (DHA-STS), which is an enzyme that is believed to have a pivotal role in regulating the biosynthesis of the oestrogenic steroid androstenediol^{8,9}. Also, there is now evidence to suggest that androstenediol may be of even greater importance as a promoter of breast tumour growth¹⁰. EMATE is also active *in vivo* as almost complete inhibition of rat liver E1-STS (99%) and DHA-STS (99%) activities resulted when it is administered either orally or subcutaneously¹¹. In addition, EMATE has been shown to have a memory enhancing effect in rats¹⁴. Studies in mice have suggested an association between DHA-STS activity and the regulation of part of the immune response. It is thought that this may also occur in humans^{15,16}. The bridging O-atom of the sulphamate moiety in EMATE is important for inhibitory activity. Thus, when the 3-O-atom is replaced by other heteroatoms (Figure 1) as in oestrone-3-N-sulphamate (4) and oestrone-3-S-sulphamate (5), these analogues are weaker non-time-dependent inactivators¹².

Although optimal potency for inhibition of E1-STS may have been attained in EMATE, it is possible that oestrone may be released during sulphatase inhibition^{8,12}, and that EMATE and its oestradiol congener may possess oestrogenic activity¹³.

The present invention seeks to provide novel compounds suitable for the inhibition of E1-STS but preferably wherein those compounds have no, or a minimal, oestrogenic effect.

5 According to a first aspect of the present invention there is provided a sulphamate compound suitable for use as an inhibitor of oestrone sulphatase, wherein the compound has the Formula I; wherein A is a first group; B is an aryl ring structure having at least 4 carbon atoms in the ring and wherein the ring B is substituted in at least the 2 position and/or the 4 position with an atom or group other than H; X is a sulphamate group; wherein group A and ring B together are capable of mimicking the A and B rings of
10 oestrone; and wherein group A is attached to at least one carbon atom in ring B.

The term "mimic" as used herein means having a similar or different structure but having a similar functional effect. In other words, group A and ring B together of the compounds of the present invention are bio-isosteres of the A and B rings of oestrone.

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A key advantage of the present invention is that the sulphamate compounds of the present invention can act as E1-STS inhibitors.

20 Another advantage of the compounds of the present invention is that they may be potent *in vivo* and that they may have less oestrogenic activity than the known compounds and can therefore be deemed to be a "non-oestrogenic compound". The term "non-oestrogenic compound" as used herein means a compound exhibiting no or substantially no oestrogenic activity.

25 The present invention therefore provides sulphamate compounds which may have a reduced oestrogenic activity.

Another advantage is that the compounds may not be capable of being metabolised to compounds which display or induce hormonal activity.

The compounds of the present invention are also advantageous in that they may be orally active.

- 5 The compounds of the present invention are further advantageous in that they may have an irreversible effect.

In a preferred embodiment, the sulphamate compounds of the present invention are useful for the treatment of breast cancer.

10

In addition, the sulphamate compounds of the present invention are useful for the treatment of non-malignant conditions, such as the prevention of auto-immune diseases, particularly when pharmaceuticals may need to be administered from an early age.

- 15 The sulphamate compounds of the present invention are also believed to have therapeutic uses other than for the treatment of endocrine-dependent cancers, such as the treatment of autoimmune diseases.

Preferably, the sulphamate group is at position 3 of the ring B.

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Preferably, the ring B has six carbon atoms in the ring.

- Preferably, the compound has the Formula II; wherein X is the sulphamate group; A is the first group; R_1 and/or R_2 is a substituent other than H; wherein R_1 and R_2 may be the same or different but not both being H; and wherein optionally group A is attached to at least one other carbon atom in ring B.
- 25

Preferably, group A is additionally attached to the carbon atom at position 1 of the ring B.

Preferably, group A and ring B are a steroid ring structure or a substituted derivative thereof.

- 5 Preferably, the compound has the Formula IV; wherein X is the sulphamate group; R_1 and/or R_2 is a substituent other than H; wherein R_1 and R_2 may be the same or different but not both being H; and wherein Y is a suitable linking group.

10 Suitable linking groups for Y include groups made up of at least any one or more of C, O, N, and S. The linking groups can also comprise H. The linking group may also increase the size of the ring (i.e. the D ring). Preferably, however, the D ring comprising Y is a five-membered ring.

Preferably, Y is $-\text{CH}_2-$ or $-\text{C}(\text{O})-$.

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Preferably, Y is $-\text{C}(\text{O})-$.

- 20 Preferably, the compound has the Formula V; wherein X is the sulphamate group; R_1 and/or R_2 is a substituent other than H; and wherein R_1 and R_2 may be the same or different but not both being H.

The term "sulphamate" as used herein includes an ester of sulphamic acid, or an ester of an N-substituted derivative of sulphamic acid, or a salt thereof.

- 25 Preferably, the sulphamate group has the Formula III.

In Formula III, each of R_3 and R_4 is independently selected from H or a hydrocarbyl group.

The term "hydrocarbyl group" as used herein means a group comprising at least C and H and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, an alkyl group, a cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of substituents may form a cyclic group. If the hydrocarbyl group comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group. Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in the art and include, for instance, sulphur, nitrogen and oxygen. A non-limiting example of a hydrocarbyl group is an acyl group.

In one preferred embodiment of the present invention, the hydrocarbyl group for the sulphamate group is a hydrocarbon group.

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Here the term "hydrocarbon" means any one of an alkyl group, an alkenyl group, an alkynyl group, which groups may be linear, branched or cyclic, or an aryl group. The term hydrocarbon also includes those groups but wherein they have been optionally substituted. If the hydrocarbon is a branched structure having substituent(s) thereon, then the substitution may be on either the hydrocarbon backbone or on the branch; alternatively the substitutions may be on the hydrocarbon backbone and on the branch.

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Preferably, R_3 and R_4 are independently selected from H or alkyl, cycloalkyl, alkenyl and aryl, or together represent alkylene, wherein the or each alkyl or cycloalkyl or alkenyl or optionally contain one or more hetero atoms or groups.

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When substituted, the N-substituted compounds of this invention may contain one or two N-alkyl, N-alkenyl, N-cycloalkyl or N-aryl substituents, preferably containing or each containing a maximum of 10 carbon atoms. When R_3 and/or R_4 is alkyl, the preferred values are those where R_3 and R_4 are each independently selected from lower alkyl groups containing from 1 to 5 carbon atoms, that is to say methyl, ethyl, propyl etc. Preferably

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R₃ and R₄ are both methyl. When R₃ and/or R₄ is aryl, typical values are phenyl and tolyl (-PhCH₃; *o*-, *m*- or *p*-). Where R₃ and R₄ represent cycloalkyl, typical values are cyclopropyl, cyclopentyl, cyclohexyl etc. When joined together R₃ and R₄ typically represent an alkylene group providing a chain of 4 to 6 carbon atoms, optionally interrupted by one or more hetero atoms or groups, e.g. -O- or -NH- to provide a 5-, 6- or 7- membered heterocycle, e.g. morpholino, pyrrolidino or piperidino.

Within the values alkyl, cycloalkyl, alkenyl and aryl we include substituted groups containing as substituents therein one or more groups which do not interfere with the sulphatase inhibitory activity of the compound in question. Exemplary non-interfering substituents include hydroxy, amino, halo, alkoxy, alkyl and aryl.

In some preferred embodiments, at least one of R₃ and R₄ is H.

In some further preferred embodiments, each of R₃ and R₄ is H.

Preferably, each of R₁ and R₂ is independently selected from H, alkyl, cycloalkyl, alkenyl, aryl, substituted alkyl, substituted cycloalkyl, substituted alkenyl, substituted aryl, any other suitable hydrocarbyl group, a nitrogen containing group, a S containing group, a carboxy containing group.

Likewise, here, the term "hydrocarbyl group" means a group comprising at least C and H and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, an alkyl group, a cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of substituents may form a cyclic group. If the hydrocarbyl group comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group. Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in

the art and include, for instance, sulphur, nitrogen and oxygen. A non-limiting example of a hydrocarbyl group is an acyl group.

Preferably, each of R_1 and R_2 is independently selected from H, C_{1-6} alkyl, C_{1-6} cycloalkyl, C_{1-6} alkenyl, substituted C_{1-6} alkyl, substituted C_{1-6} cycloalkyl, substituted C_{1-6} alkenyl, substituted aryl, a nitrogen containing group, a S containing group, or a carboxy group having from 1-6 carbon atoms.

Likewise, here within the values alkyl, cycloalkyl, alkenyl and aryl we include substituted groups containing as substituents therein one or more groups which do not interfere with the sulphatase inhibitory activity of the compound in question. Exemplary non-interfering substituents include hydroxy, amino, halo, alkoxy, alkyl and aryl.

Preferably, each of R_1 and R_2 is independently selected from H, C_{1-6} alkyl, C_{1-6} alkenyl, a nitrogen containing group, or a carboxy group having from 1-6 carbon atoms.

Preferably, each of R_1 and R_2 is independently selected from H, C_{1-6} alkyl, C_{1-6} alkenyl, NO_2 , or a carboxy group having from 1-6 carbon atoms.

Preferably, each of R_1 and R_2 is independently selected from H, C_3 alkyl, C_3 alkenyl, NO_2 , or H_3CHO .

Preferably, the compound is any one of the Formulae V - IX.

Preferably, for some applications, the compound is further characterised by the feature that if the sulphonate group were to be substituted by a sulphate group to form a sulphate derivative, then the sulphate derivative would be hydrolysable by an enzyme having steroid sulphonatase (E.C. 3.1.6.2) activity - i.e. when incubated with steroid sulphonatase EC 3.1.6.2 at pH 7.4 and 37°C.

In one preferred embodiment, if the sulphamate group of the compound were to be replaced with a sulphate group to form a sulphate compound then that sulphate compound would be hydrolysable by an enzyme having steroid sulphatase (E.C. 3.1.6.2) activity and
5 would yield a K_m value of less than 50 mmolar when incubated with steroid sulphatase EC 3.1.6.2 at pH 7.4 and 37° C.

In another preferred embodiment, if the sulphamate group of the compound were to be replaced with a sulphate group to form a sulphate compound then that sulphate compound
10 would be hydrolysable by an enzyme having steroid sulphatase (E.C. 3.1.6.2) activity and would yield a K_m value of less than 50 μ molar when incubated with steroid sulphatase EC 3.1.6.2 at pH 7.4 and 37° C.

In a highly preferred embodiment, the compound of the present invention is not
15 hydrolysable by an enzyme having steroid sulphatase (E.C. 3.1.6.2) activity.

Thus, the present invention provides novel sulphamate compounds.

Preferably the group A and the ring B together--hereinafter referred to as "group A/ring B
20 combination" --will contain, inclusive of all substituents, a maximum of about 50 carbon atoms, more usually no more than about 30 to 40 carbon atoms.

A preferred group A/ring B combination has a steroidal ring structure, that is to say a cyclopentanophenanthrene skeleton. Preferably, the sulphonyl or substituted sulphonyl
25 group is attached to that skeleton in the 3-position.

Thus, according to a preferred embodiment, the group A/ring B combination is a substituted or unsubstituted, saturated or unsaturated steroid nucleus.

A suitable steroid nucleus is a substituted (i.e. substituted in at least the 2 and/or 4 position and optionally elsewhere in the steroid nucleus) derivative of any one of: oestrone, 2-OH-oestrone, 2-methoxy-oestrone, 4-OH-oestrone, 6a-OH-oestrone, 7a-OH-oestrone, 16a-OH-oestrone, 16b-OH-oestrone, oestradiol, 2-OH-17b-oestradiol, 2-methoxy-17b-oestradiol, 4-OH-17b-oestradiol, 6a-OH-17b-oestradiol, 7a-OH-17b-oestradiol, 16a-OH-17a-oestradiol, 16b-OH-17a-oestradiol, 16b-OH-17b-oestradiol, 17a-oestradiol, 17b-oestradiol, 17a-ethinyl-17b-oestradiol, oestriol, 2-OH-oestriol, 2-methoxy-oestriol, 4-OH-oestriol, 6a-OH-oestriol, 7a-OH-oestriol, dehydroepiandrosterone, 6a-OH-dehydroepiandrosterone, 7a-OH-dehydroepiandrosterone, 16a-OH-dehydroepiandrosterone, 16b-OH-dehydroepiandrosterone.

In general terms the group A/ring B combination may contain a variety of non-interfering substituents. In particular, the group A/ring B combination may contain one or more hydroxy, alkyl especially lower (C_1-C_6) alkyl, e.g. methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, n-pentyl and other pentyl isomers, and n-hexyl and other hexyl isomers, alkoxy especially lower (C_1-C_6) alkoxy, e.g. methoxy, ethoxy, propoxy etc., alkenyl, e.g. ethenyl, or halogen, e.g. fluoro substituents.

The group A/ring B combination may even be a non-steroidal ring system.

A suitable non-steroidal ring system is a substituted (i.e. substituted in at least the 2 and/or 4 position and optionally elsewhere in the ring system) derivative of any one of: diethylstilboestrol, stilboestrol.

When substituted, the N-substituted compounds of this invention may contain one or two N-alkyl, N-alkenyl, N-cycloalkyl or N-aryl substituents, preferably containing or each containing a maximum of 10 carbon atoms.

When R_1 and/or R_2 and/or R_3 and/or R_4 is alkyl, the preferred values are those where each of R_1 and R_2 and R_3 and R_4 is independently selected from lower alkyl groups containing from 1 to 6 carbon atoms, that is to say methyl, ethyl, propyl etc.

- 5 When R_1 and/or R_2 and/or R_3 and/or R_4 is aryl, typical groups are phenyl and tolyl ($-\text{PhCH}_3$; *o*-, *m*- or *p*-).

Where R_1 and/or R_2 and/or R_3 and/or R_4 represent cycloalkyl, typical values are cyclopropyl, cyclopentyl, cyclohexyl etc.

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When joined together R_3 and R_4 typically represent an alkylene group providing a chain of 4 to 6 carbon atoms, optionally interrupted by one or more hetero atoms or groups, e.g. -O- or -NH- to provide a 5-, 6- or 7- membered heterocycle, e.g. morpholino, pyrrolidino or piperidino.

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Within the values alkyl, cycloalkyl, alkenyl and aryl we include substituted groups containing as substituents therein one or more groups which do not interfere with the sulphatase inhibitory activity of the compound in question. Examples of non-interfering substituents include hydroxy, amino, halo, alkoxy, alkyl and aryl.

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We have also surprisingly found that when the compound has the Formula IV where $Y = -\text{CH}_2-$ it is not necessary for the compound to be substituted in the 2 and 4 ring positions, ie R_1 and R_2 may both be hydrogen. In one embodiment of this aspect, any of the ring positions (including R_1 and R_2 , but excluding Y) may be substituted.

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Thus, according to another aspect of the present invention there is provided a sulphamate compound suitable for use as an inhibitor of oestrone sulphatase wherein the compound has the Formula X and wherein X is a sulphamate group, and Y is CH_2 and optionally any other H attached directly to the ring system is substituted by another group.

X may be as described above.

Any replacement for H on the ring system may be any one of the substituents described
5 above in relation to R_1 and R_2 .

In an especially preferred embodiment there is no substitution on the ring system, ie a compound of Formula IV where Y is $-CH_2-$ and R_1 and R_2 are both H.

10 According to a second aspect of the present invention there is provided a sulphamate compound according to the present invention for use as a pharmaceutical.

According to a third aspect of the present invention there is provided a sulphamate compound according to the present invention for inhibiting oestrone sulphotase.

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According to a fourth aspect of the present invention there is provided a pharmaceutical composition comprising a sulphamate compound according to the present invention; and a pharmaceutically acceptable carrier, excipient, adjuvant or diluent.

20 According to a fifth aspect of the present invention there is provided the use of a sulphamate compound according to the present invention in the manufacture of a pharmaceutical for inhibiting oestrone sulphotase.

The sulphamate compounds of the present invention may be prepared by reacting an
25 appropriate alcohol with a sulfamoyl chloride, $R_3R_4NSO_2Cl$.

Preferred conditions for carrying out the reaction are as follows.

Sodium hydride and a sulfamoyl chloride are added to a stirred solution of the alcohol in anhydrous dimethyl formamide at 0°C. Subsequently, the reaction is allowed to warm to room temperature whereupon stirring is continued for a further 24 hours. The reaction mixture is poured onto a cold saturated solution of sodium bicarbonate and the resulting aqueous phase is extracted with dichloromethane. The combined organic extracts are dried over anhydrous MgSO₄. Filtration followed by solvent evaporation *in vacuo* and co-evaporated with toluene affords a crude residue which is further purified by flash chromatography.

- 10 Preferably, the alcohol is derivatised, as appropriate, prior to reaction with the sulfamoyl chloride. Where necessary, functional groups in the alcohol may be protected in known manner and the protecting group or groups removed at the end of the reaction.

For pharmaceutical administration, the steroid sulphatase inhibitors of this invention can be formulated in any suitable manner utilising conventional pharmaceutical formulating techniques and pharmaceutical carriers, adjuvants, excipients, diluents etc. and usually for parenteral administration. Approximate effective dose rates are in the range 100 to 800 mg/day depending on the individual activities of the compounds in question and for a patient of average (70Kg) bodyweight. More usual dosage rates for the preferred and more active compounds will be in the range 200 to 800 mg/day, more preferably, 200 to 500 mg/day, most preferably from 200 to 250 mg/day. They may be given in single dose regimes, split dose regimes and/or in multiple dose regimes lasting over several days. For oral administration they may be formulated in tablets, capsules, solution or suspension containing from 100 to 500 mg of compound per unit dose. Alternatively and preferably the compounds will be formulated for parenteral administration in a suitable parenterally administrable carrier and providing single daily dosage rates in the range 200 to 800 mg, preferably 200 to 500, more preferably 200 to 250 mg. Such effective daily doses will, however, vary depending on inherent activity of the active ingredient and on the

bodyweight of the patient, such variations being within the skill and judgement of the physician.

5 For particular applications, it is envisaged that the steroid sulphatase inhibitors of this invention may be used in combination therapies, either with another sulphatase inhibitor, or, for example, in combination with an aromatase inhibitor, such as for example, 4-hydroxyandrostenedione (4-OHA).

10 In summation, the present invention provides novel compounds for use as steroid sulphatase inhibitors, and pharmaceutical compositions containing them.

The present invention will now be described only by way of example with reference to the accompanying drawings in which:-

15 Figure 1 shows the known structures of oestrone (1), oestrone sulphate (2), EMATE (3) and steroid sulphamates (4-5);

Figure 2 shows a compound of the Formula I;

20 Figure 3 shows a compound of the Formula II;

Figure 4 shows a compound of the Formula III;

Figure 5 shows a compound of the Formula IV;

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Figure 6 shows a compound of the Formula V;

Figure 7 shows a compound of the Formula VI;

Figure 8 shows a compound of the Formula VII;

5 Figure 9 shows a compound of the Formula VIII;

Figure 10 shows a compound of the Formula IX;

Figure 11 shows a compound of the Formula X;

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Figure 12 shows one embodiment of a method of preferring compounds of the present invention;

Figure 13 shows another embodiment of a method of preferring compounds of the present
15 invention;

Figure 14 shows yet another embodiment of a method of preferring compounds of the present invention;

20 Figure 15 shows a further embodiment of a method of preferring compounds of the present invention;

Figure 16 shows a graph illustrating the *in vivo* inhibition of oestrone sulphatase by NOMATE (0.1 mg/Kg/day for five days); and

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Figure 17 shows a graph illustrating the lack of effect of NOMATE (0.1 mg/Kg/day for five days) on uterine weights in ovariectomised rats.

The invention will now be described only by way of Examples.

Example 1 - Preparative Methods

The preparation of various compounds in accordance with the present invention is illustrated in Figures 12 to 15. In these Figures, the curved lines attached to the phenyl rings represent the remainder of the ringed structure.

Example 1 - *In Vitro* Inhibition

The ability of compounds to inhibit oestrone sulphatase activity was assessed using either intact MCF-7 breast cancer cells or placental microsomes as previously described¹¹.

In this regard, the teachings of that earlier reference¹¹ are as follows:

Inhibition of Steroid Sulphatase Activity in MCF-7 cells by oestrone-3-sulphamate

Steroid sulphatase is defined as: Steryl Sulphatase EC 3.1.6.2.

Steroid sulphatase activity was measured *in vitro* using intact MCF-7 human breast cancer cells. This hormone dependent cell line is widely used to study the control of human breast cancer cell growth. It possesses significant steroid sulphatase activity (MacIndoe et al. *Endocrinology*, 123, 1281-1287 (1988); Purohit & Reed, *Int. J. Cancer*, 50, 901-905 (1992)) and is available in the U.S.A. from the American Type Culture Collection (ATCC) and in the U.K. (e.g. from The Imperial Cancer Research Fund). Cells were maintained in Minimal Essential Medium (MEM) (Flow Laboratories, Irvine, Scotland) containing 20 mM HEPES, 5% foetal bovine serum, 2 mM glutamine, non-essential

amino acids and 0.075% sodium bicarbonate. Up to 30 replicate 25 cm² tissue culture flasks were seeded with approximately 1×10^5 cells/flask using the above medium. Cells were grown to 80% confluency and medium was changed every third day.

- 5 Intact monolayers of MCF-7 cells in triplicate 25 cm² tissue culture flasks were washed with Earle's Balanced Salt Solution (EBSS from ICN Flow, High Wycombe, U.K.) and incubated for 3-4 hours at 37°C with 5 pmol (7×10^5 dpm) [6,7-³H]oestrone-3-sulphate (specific activity 60 Ci/mmol from New England Nuclear, Boston, Mass., U.S.A.) in serum-free MEM (2.5 ml) together with oestrone-3-sulphamate (11 concentrations: 0; 10 1fM; 0.01pM; 0.1pM; 1pM; 0.01nM; 0.1nM; 1nM; 0.01mM; 0.1mM; 1mM). After incubation each flask was cooled and the medium (1 ml) was pipetted into separate tubes containing [¹⁴C]oestrone (7×10^3 dpm) (specific activity 97 Ci/mmol from Amersham International Radiochemical Centre, Amersham, U.K.). The mixture was shaken thoroughly for 30 seconds with toluene (5 ml). Experiments showed that >90% 15 [¹⁴C]oestrone and <0.1% [³H]oestrone-3-sulphate was removed from the aqueous phase by this treatment. A portion (2 ml) of the organic phase was removed, evaporated and the ³H and ¹⁴C content of the residue determined by scintillation spectrometry. The mass of oestrone-3-sulphate hydrolysed was calculated from the ³H counts obtained (corrected for the volumes of the medium and organic phase used, and for recovery of [¹⁴C]oestrone 20 added) and the specific activity of the substrate. Each batch of experiments included incubations of microsomes prepared from a sulphatase-positive human placenta (positive control) and flasks without cells (to assess apparent non-enzymatic hydrolysis of the substrate). The number of cell nuclei per flask was determined using a Coulter Counter after treating the cell monolayers with Zaponin. One flask in each batch was used to 25 assess cell membrane status and viability using the Trypan Blue exclusion method (Phillips, H.J. (1973) In: *Tissue culture and applications*, [eds: Kruse, D.F. & Patterson, M.K.]; pp. 406-408; Academic Press, New York).

Results for steroid sulphatase activity are expressed as the mean \pm 1 S.D. of the total 30 product (oestrone + oestradiol) formed during the incubation period (20 hours) calculated

for 10^6 cells and, for values showing statistical significance, as a percentage reduction (inhibition) over incubations containing no oestrone-3-sulphamate. Unpaired Student's t-test was used to test the statistical significance of results.

5. Inhibition of Steroid Sulphatase Activity in Placental Microsomes by Oestrone-3-sulphamate

Sulphatase-positive human placenta from normal term pregnancies (Obstetric Ward, St. Mary's Hospital, London) were thoroughly minced with scissors and washed once with cold phosphate buffer (pH 7.4, 50 mM) then re-suspended in cold phosphate buffer (5 ml/g tissue). Homogenisation was accomplished with an Ultra-Turrax homogeniser, using three 10 second bursts separated by 2 minute cooling periods in ice. Nuclei and cell debris were removed by centrifuging (4°C) at 2000g for 30 minutes and portions (2 ml) of the supernatant were stored at -20°C . The protein concentration of the supernatants was determined by the method of Bradford (*Anal. Biochem.*, 72, 248-254 (1976)).

Incubations (1 ml) were carried out using a protein concentration of 100 mg/ml, substrate concentration of 20 mM [6,7- ^3H]oestrone-3-sulphate (specific activity 60 Ci/mmol from New England Nuclear, Boston, Mass., U.S.A.) and an incubation time of 20 minutes at 37°C . If necessary eight concentrations of compounds are employed: 0 (i.e. control); 0.05mM; 0.1mM; 0.2mM; 0.4mM; 0.6mM; 0.8mM; 1.0mM. After incubation each sample was cooled and the medium (1 ml) was pipetted into separate tubes containing [^{14}C]oestrone (7×10^3 dpm) (specific activity 97 Ci/mmol from Amersham International Radiochemical Centre, Amersham, U.K.). The mixture was shaken thoroughly for 30 seconds with toluene (5 ml). Experiments showed that $>90\%$ [^{14}C]oestrone and $<0.1\%$ [^3H]oestrone-3-sulphate was removed from the aqueous phase by this treatment. A portion (2 ml) of the organic phase was removed, evaporated and the ^3H and ^{14}C content of the residue determined by scintillation spectrometry. The mass of oestrone-3-sulphate hydrolysed was calculated from the ^3H counts obtained (corrected for the volumes of the

medium and organic phase used, and for recovery of [^{14}C]oestrone added) and the specific activity of the substrate.

For the present invention, the percentage inhibition for the series of EMATE analogues tested in either MCF-7 cells or placental microsomes is shown in Table 1.

Example 2 - In Vivo Studies

Using 17-deoxy oestrone-3-O-sulphamate (NOMATE, Figure 5, Formula IV where $X = -\text{OSO}_2\text{NH}_2$, $Y = -\text{CH}_2-$ and R_1 and $R_2 = \text{H}$, and Figure 13) as a representative example, the ability of this compound to inhibit oestrone sulphatase activity *in vivo* was examined in rats. The oestrogenicity of this compound was examined in ovariectomised rats. In this model compounds which are oestrogenic stimulate uterine growth.

(i) Inhibition of oestrone sulphatase activity *in vivo*

NOMATE (0.1 mg/Kg/day for five days) was administered orally to rats with another group of animals receiving vehicle only (propylene glycol). At the end of the study samples of liver tissue were obtained and oestrone sulphatase activity assayed using ^3H oestrone sulphate as the substrate as previously described¹¹.

As shown in Figure 16, administration of this dose of NOMATE effectively inhibited oestrone sulphatase activity by 98% compared with untreated controls.

(ii) Lack of *in vivo* oestrogenicity

NOMATE (0.1 mg/Kg/day for five days) was administered orally to rats with another group of animals receiving vehicle only (propylene glycol). At the end of the study uteri
5 were obtained and weighed with the results being expressed as uterine weight/whole body weight x 100.

As shown in Figure 17, administration of NOMATE at the dose tested, but had no significant effect on uterine growth, showing that at this dose the compound is not
10 oestrogenic.

TABLE 1

Inhibition of Oestrone Sulphatase Activity in MCF-7 Cells
or Placental Microsomes by EMATE Analogues

Inhibitor	Concentration Tested (mM)	% Inhibition (Mean)	
		MCF-7 Cells	Placental Microsomes
2-n-propyl EMATE	0.1	41.1	-
	1	83.1	21.9
	10	92.2	43.2
	25	-	47.5
	50	-	61.1
	100	-	69.2
4-n-propyl EMATE	1	-	13.7
	10	-	10.2
	25	-	15.7
	50	-	16.3
	100	-	23.7
2,4-n-dipropyl EMATE	0.1	6.6	-
	1	10.6	-
2-allyl EMATE	0.01	23.2	-
	0.1	76.1	-
	1	94.2	45.6
	10	93.7	65.4
	25	-	75.3
	50	-	86.6
	100	-	89.6
4-allyl EMATE (approx 75%)	1	-	29.1
	10	-	54.2
	25	-	59.0

	50	-	65.1
	100	-	71.9
2,4-di-allyl EMATE	-	-	-
2-methoxy EMATE	0.1	96.0	-
	1	93.6	-
	10	96.2	99.0
	50	-	99.7
	100	-	99.7
2-nitro EMATE	0.05	-	44.5
	0.5	-	93.9
	5	-	99.0
	50	-	99.4
4-nitro EMATE	20	-	99.0
NOMATE	0.1	96.4	97.2
(17-deoxy EMATE)	1	99.1	99.5
	10	99.7	99.5
	25	99.7	99.7

- = not tested

- Irreversible time- and concentration-dependent inhibition is assumed for these compounds in keeping with established precedent⁸.

Other modifications of the present invention will be apparent to those skilled in the art.

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The present invention relates to a composition. In particular the present invention relates to a pharmaceutical composition - and to a class of compounds particularly useful in or as said composition.

Cancer remains a major cause of mortality in most Western countries. So far, evidence suggests that oestrogens are the major mitogens involved in promoting the growth of tumours in endocrine-dependent tissues, such as the breast and endometrium. Although plasma oestrogen concentrations are similar in women with or without breast cancer, breast tumour oestrone and oestradiol levels are significantly higher than in normal breast tissue or blood. *In situ* synthesis of oestrogen is thought to make an important contribution to the high levels of oestrogens in tumours and therefore specific inhibitors of oestrogen biosynthesis are of potential value for the treatment of endocrine-dependent tumours.

Over the past two decades, there has been considerable interest in the development of inhibitors of the aromatase pathway which converts the androgen precursor androstenedione to oestrone. However, there is now evidence that the oestrone sulphatase ("E1-STS") pathway, i.e. the hydrolysis of oestrone sulphate ("E1S") to oestrone ("E1"), as opposed to the aromatase pathway, is the major source of oestrogen in breast tumours. This theory is supported by a modest reduction of plasma oestrogen concentration in postmenopausal women with breast cancer treated by aromatase inhibitors, such as aminoglutethimide and 4-hydroxyandrostenedione and also by the fact that plasma E1S concentration in these aromatase inhibitor-treated patients remains relatively high. The long half-life of E1S in blood (10-12 h) compared with the unconjugated oestrogens (20 min) and high levels of steroid sulphatase activity in liver and, normal and malignant breast tissues, also lend support to this theory.

Singh *et al* (1997 J Steroid Biochem Mol Biol 61: 185-192), report that the major source of pro-inflammatory cytokines, such as TNF- α and IL-6 within breast tumours is not well understood but it is thought that tumour infiltrating macrophages and lymphocytes might play a role.

Singh *et al* (*ibid*) report that the release of cytokines, such as IL-6 by tumour cells is also associated with enhanced aromatase activity in breast tissue adjacent to the tumour.

5 Singh *et al* (*ibid*) also report that both TNF- α and IL-6 inhibit the growth of MCF-7 breast cancer cells *in vitro*. In addition, TNF- α has an inhibitory effect on aromatase activity measured in cultured MCF-7 breast cancer cells. Apparently, these results contrast with the marked stimulatory effect that TNF- α has on fibroblasts derived from normal and malignant breast tissues (Macdiarmaid *et al* 1994 Molec. Cell Endoc. 106: 10 17-21). In addition, when TNF- α is combined with IL-6, the inhibitory effect on aromatase activity is enhanced. The synergistic inhibitory effect of IL-6 and TNF- α on aromatase activity in MCF-7 cells also contrasts to the synergistic stimulatory effect that these cytokines have on oestrone sulphatase and oestradiol dehydrogenase activities in these cells.

15

Singh *et al* (*ibid*) also report that a significant reduction in aromatase activity is observed when conditioned media (CM) from monocytes and lymphocytes of an immunosuppressed kidney transplant patient is added to fibroblast cultures from normal breast cells compared with CM from breast cancer cells. These results suggest that the 20 reduced incidence of breast cancer in immunosuppressed kidney transplant patients could result from reduced cytokine production and thus decreased stimulation of oestrogen synthesis.

Previous studies have also shown that where CM from cultured breast cancer cells 25 stimulates aromatase activity, this CM also stimulates the activities of two main enzymes, that is oestrone sulphatase and oestradiol dehydrogenase which are also involved in breast tumour oestrogen synthesis.

Thus, there appears to be a co-ordinated mechanism for regulating the synthesis of 30 oestrogen within breast tumours that is controlled by cytokines. However, it has been postulated that any *in vivo* stimulatory effect of cytokines in inhibiting tumour growth

may be outweighed by their stimulatory effect on enzyme activity associated with oestrogen synthesis (Duncan and Reed 1995 J Steroid Biochem Molec Biol 55:565-572).

5 Singh *et al (ibid)* state that while cytokines such as TNF- α and IL-6 have been shown to play an important role in regulating the activities of enzymes involved in oestrogen synthesis, it is likely that other cytokines and mediators of the inflammatory response are capable of modulating oestrogen synthesis in normal and malignant breast tissue.

10 Thus, cancer therapies developed so far have included blocking the action or synthesis of hormones to inhibit the growth of hormone-dependent tumours. However, more aggressive chemotherapy is currently employed for the treatment of hormone-independent tumours.

15 Hence, the development of a pharmaceutical for anti-cancer treatment of hormone dependent and/or hormone independent tumours, yet lacking some or all of the side-effects associated with chemotherapy, would represent a major therapeutic advance.

20 In fact, Singh *et al (ibid)* state that "*by understanding the complex mechanisms which govern oestrogen synthesis, it should be possible to devise better preventative and therapeutic strategies*" against cancers - especially breast cancer.

The present invention seeks to provide a composition suitable for use in the treatment of cancers and, especially, breast cancer.

25 According to a first aspect of the present invention there is provided a composition comprising i) a compound comprising a sulphonamide group ("a sulphonamide compound"); and ii) a biological response modifier.

30 According to a second aspect of the present invention there is provided the use of a composition according to the present invention in the manufacture of a medicament to prevent and/or inhibit tumour growth.

According to a third aspect of the present invention there is provided the use of a composition according to the present invention in the manufacture of a medicament to do any one or more of: prevent or suppress glucose uptake by a tumour; prevent and/or inhibit tumour angiogenesis; disrupt microtubules; induce apoptosis.

5

According to a fourth aspect of the present invention there is provided the use of a sulphamate compound comprising a steroidal component and an oxyhydrocarbyl group ("oxyhydrocarbyl steroidal sulphamate compound") in the manufacture of a medicament to do any one or more of: prevent or suppress glucose uptake by a tumour; prevent
10 and/or inhibit tumour angiogenesis; disrupt microtubules; induce apoptosis.

According to a fifth aspect of the present invention there is provided the composition of the present invention for use in medicine.

15 According to a sixth aspect of the present invention there is provided a method of treatment comprising administering to a subject in need of treatment a composition according to the present invention.

According to a seventh aspect of the present invention there is provided a method of
20 treatment comprising administering to a subject in need of treatment a composition according to the present invention or an oxyhydrocarbyl steroidal sulphamate compound according to the present invention in order to prevent or suppress glucose uptake by a tumour; and/or prevent and/or inhibit tumour angiogenesis; and/or disrupt microtubules; and/or induce apoptosis.

25

According to an eighth aspect of the present invention there is provided a kit comprising a part i) containing a compound comprising a sulphamate group ("a sulphamate compound"); and a part ii) containing a biological response modifier. The parts of the kit may be independently held in one or more containers - such as bottles, syringes,
30 plates, wells, blister pack etc.

The present invention is advantageous in that it provides a composition suitable for use in the treatment of cancers and, especially, breast cancer.

In addition, the present invention is advantageous in that it provides a compound that is suitable for use in the treatment of cancers such as breast cancer, ovarian cancer, endometrial cancer, sarcomas, melanomas, prostate cancer etc. - especially, breast cancer.

Another advantage of the compositions of the present invention is that they may be more potent *in vivo* than the sulphamate compounds alone or the biological response modifier alone. Moreover, in some aspects the combination of sulphamate compounds and the biological response modifier is more potent than one would expect from the potency of the compound alone i.e. this is a synergistic relationship between them.

In accordance with the present invention the composition of the present invention may comprise more than one biological response modifier.

The term biological response modifier ("BRM") includes cytokines, immune modulators, growth factors, haematopoiesis regulating factors, colony stimulating factors, chemotactic, haemolytic and thrombolytic factors, cell surface receptors, ligands, leukocyte adhesion molecules, monoclonal antibodies, preventative and therapeutic vaccines, hormones, extracellular matrix components, fibronectin, etc.

BRMs may play a role in modulating the immune and inflammatory response in disorders. Examples of BRMs include: Tumour Necrosis Factor (TNF), granulocyte colony stimulating factor, erythropoietin, insulin-like growth factor (IGF), epidermal growth factor (EGF), transforming growth factor (TGF), platelet-derived growth factor (PDGF), interferons (IFNs), interleukins, tissue plasminogen activators, P-, E- or L- Selectins, ICAM-1, VCAM, Selectins, addressins etc.

Preferably, the biological response modifier is a cytokine.

A cytokine is a molecule - often a soluble protein - that allows immune cells to communicate with each other. These molecules exert their biological functions through specific receptors expressed on the surface of target cells. Binding of the receptors triggers the release of a cascade of biochemical signals which profoundly affect the behaviour of the cell bearing the receptor (Poole, S 1995 TibTech 13: 81-82). Many cytokines and their receptors have been identified at the molecular level (Paul and Sedar 1994, Cell 76: 241-251) and make suitable molecules of therapeutic value as well as therapeutic targets in their own right.

More details on cytokines can be found in Molecular Biology and Biotechnology (Pub. VCH, Ed. Meyers, 1995, pages 202, 203, 394, 390, 475, 790).

Examples of cytokines include: interleukins (IL) - such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-19; Tumour Necrosis Factor (TNF) - such as TNF- α ; Interferon alpha, beta and gamma; TGF- β .

For the present invention, preferably the cytokine is tumour necrosis factor (TNF).

More preferably the cytokine is TNF- α .

20

TNF is a cytokine produced by macrophages and lymphocytes which mediates inflammatory and immunopathological responses. TNF has been implicated in the progression of diseases which include but are not limited to immunomodulation disorder, infection, cell proliferation, angiogenesis (neovascularisation), tumour metastasis, apoptosis, sepsis, and endotoxaemia.

25

The necrotising action of TNF *in vivo* mainly relates to capillary injury. TNF causes necrosis not only in tumour tissue but also in granulation tissue. It causes morphological changes in growth inhibition of and cytotoxicity against cultured vascular endothelial cells (Haranka et al 1987 Ciba Found Symp 131: 140-153).

30

For the preferred aspect of the present invention, the TNF may be any type of TNF - such as TNF- α , TNF- β , including derivatives or mixtures thereof.

5 Teachings on TNF may be found in the art - such as WO-A-98/08870 and WO-A-98/13348.

The TNF can be prepared chemically or it can be extracted from sources. Preferably, the TNF is prepared by use of recombinant DNA techniques.

10 With this aspect of the present invention the compositions of the present invention are more potent *in vivo* than the sulphamate compounds alone or TNF alone. Moreover, in some aspects the combination of sulphamate compounds and TNF is more potent than one would expect from the potency of the compound alone i.e. this is a synergistic relationship between them.

15

In accordance with the present invention the composition of the present invention may comprise more than one sulphamate compound.

20 The term "sulphamate compound" means a compound comprising at least one sulphamate group.

Preferably, if the sulphamate group on the sulphamate compound were to be replaced with a sulphate group to form a sulphate compound then the sulphate compound would be hydrolysable by a steroid sulphotase enzyme (E.C.3.1.6.2).

25

Preferably if the sulphamate group on the sulphamate compound were to be replaced with a sulphate group to form a sulphate compound and the sulphate compound were to be incubated with a steroid sulphotase enzyme (E.C.3.1.6.2) at a pH 7.4 and 37°C it would provide a K_m value of less than 50 mM.

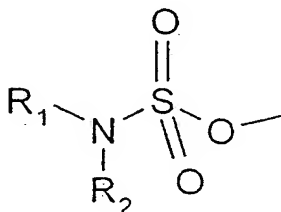
Preferably if the sulphamate group on the sulphamate compound were to be replaced with a sulphate group to form a sulphate compound and the sulphate compound were to be incubated with a steroid sulphatase enzyme (E.C.3.1.6.2) at a pH 7.4 and 37°C it would provide a K_m value of less than 50 μ M.

5

The term "sulphamate" includes an ester of sulphamic acid, or an ester of an N-substituted derivative of sulphamic acid, or a salt thereof.

Preferably, the sulphamate group of the sulphamate compound has the formula:

10



wherein each of R_1 and R_2 is independently selected from H or a hydrocarbyl group.

15 The term "hydrocarbyl group" as used herein means a group comprising at least C and H and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, a hydrocarbon group, an N-acyl group, a cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of substituents may form a cyclic group. If the hydrocarbyl group
20 comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group. Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in the art and include, for instance, sulphur, nitrogen and oxygen.

25

In one preferred embodiment of the present invention, the hydrocarbyl group is a hydrocarbon group.

Here the term "hydrocarbon" means any one of an alkyl group, an alkenyl group, an alkynyl group, an acyl group, which groups may be linear, branched or cyclic, or an aryl group. The term hydrocarbon also includes those groups but wherein they have been optionally substituted. If the hydrocarbon is a branched structure having
5 substituent(s) thereon, then the substitution may be on either the hydrocarbon backbone or on the branch; alternatively the substitutions may be on the hydrocarbon backbone and on the branch.

Preferably, R_1 and R_2 are independently selected from H or alkyl, cycloalkyl, alkenyl and
10 aryl, or together represent alkylene, wherein the or each alkyl or cycloalkyl or alkenyl or optionally contain one or more hetero atoms or groups.

When substituted, the N-substituted compounds of this invention may contain one or two N-alkyl, N-alkenyl, N-cycloalkyl, N-acyl, or N-aryl substituents, preferably containing or
15 each containing a maximum of 10 carbon atoms. When R_1 and/or R_2 is alkyl, the preferred values are those where R_1 and R_2 are each independently selected from lower alkyl groups containing from 1 to 5 carbon atoms, that is to say methyl, ethyl, propyl etc. Preferably R_1 and R_2 are both methyl. When R_1 and/or R_2 is aryl, typical values are phenyl and tolyl (-PhCH₃; *o*-, *m*- or *p*-). Where R_1 and R_2 represent cycloalkyl, typical
20 values are cyclopropyl, cyclopentyl, cyclohexyl etc. When joined together R_1 and R_2 typically represent an alkylene group providing a chain of 4 to 6 carbon atoms, optionally interrupted by one or more hetero atoms or groups, e.g. -O- or -NH- to provide a 5-, 6- or 7- membered heterocycle, e.g. morpholino, pyrrolidino or piperidino.

25 Within the values alkyl, cycloalkyl, alkenyl, acyl and aryl we include substituted groups containing as substituents therein one or more groups which do not interfere with the sulphatase inhibitory activity of the compound in question. Exemplary non-interfering substituents include hydroxy, amino, halo, alkoxy, alkyl and aryl. A non-limiting example of a hydrocarbyl group is an acyl group.

30

In some preferred embodiments, at least one of R_1 and R_2 is H.

Preferably the sulphamate compound is a cyclic compound. In this regard, the sulphamate compound can be a single ring compound or a polycyclic compound. Here, the term "polycyclic" includes fused and non-fused ring structures including combinations thereof.

5

Thus, preferably the sulphamate compound is of the formula



10 wherein E is a sulphamate group and wherein G is a cyclic group.

The cyclic group may be a single ring or it is a polycyclic ring structure.

In one aspect, the cyclic group may contain any one or more of C, H, O, N, P, halogen
15 (including Cl, Br and I), S and P.

At least one of the cyclic groups may be a heterocyclic group (a heterocycle) or a non-heterocyclic group.

20 At least one of the cyclic groups may be a saturated ring structure or an unsaturated ring structure (such as an aryl group).

Preferably, at least one of the cyclic groups is an aryl ring.

25 Preferably, the sulphamate group is linked to the aryl ring.

If the cyclic group is polycyclic some or all of the ring components of the sulphamate compound may be fused together or joined *via* one or more suitable spacer groups.

30 Thus, in accordance with one aspect of the present invention, preferably the sulphamate compound is a polycyclic compound.

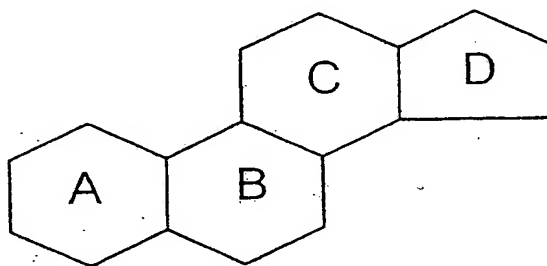
Preferably the polycyclic compound will contain, inclusive of all substituents, no more than 50 about carbon atoms, more usually no more than about 30 to 40 carbon atoms.

The polycyclic compound can comprise at least two ring components, or least three ring components, or least four ring components.

Preferably, the polycyclic compound comprises four ring components.

Preferred polycyclic compounds have a steroidal ring component - that is to say a cyclopentanophenanthrene skeleton, or bio-isosteres thereof.

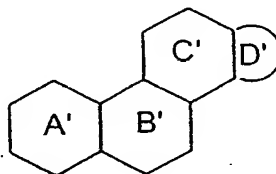
As is well known in the art, a classical steroidal ring structure has the generic formula of:



In the above formula, the rings have been labelled in the conventional manner.

An example of a bio-isostere is when any one or more of rings A, B, C and D is a heterocyclic ring and/or when any one or more of rings A, B, C and D has been substituted and/or when any one or more of rings A, B, C and D has been modified; but wherein the bio-isostere in the absence of the sulphamate group has steroidal properties.

In this regard, the structure of a preferred polycyclic compound can be presented as:



wherein each ring A', B', C' and D' independently represents a heterocyclic ring or a non-heterocyclic ring, which rings may be independently substituted or unsubstituted, saturated or unsaturated.

- 5 By way of example, any one or more of rings A', B', C' and D' may be independently substituted with suitable groups - such as an alkyl group, an allyl group, an hydroxy group, a halo group, a hydrocarbyl group, an oxyhydrocarbyl group etc.

10 An example of D' is a five or six membered non-heterocyclic ring having at least one substituent.

In one preferred embodiment, the ring D' is substituted with a ethynyl group.

15 If any one of rings A', B', C' and D' is a heterocyclic ring, then preferably that heterocyclic ring comprises a combination of C atoms and at least one N atom and/or at least one O atom. Other heterocyclic atoms may be present in the ring.

Examples of suitable, preferred steroidal nuclei rings A'-D' of the compounds of the present invention include rings A-D of oestrone and dehydroepiandrosterone.

20

Preferred steroidal nuclei rings A'-D' of the compounds of the present invention include rings A-D of:

oestrones and substituted oestrones, viz:

25

oestrone

2-OH-oestrone

2-alkoxy-oestrone (such as C₁₋₆ alkoxy-oestrone, such as 2-methoxy-oestrone)

4-OH-oestrone

6 α -OH-oestrone

30

7 α -OH-oestrone

16 α -OH-oestrone

16 β -OH-oestrone

oestradiols and substituted oestradiols, viz:

- 2-OH-17 β -oestradiol
- 2-alkoxy-17 β -oestradiol (such as C₁₋₆ alkoxy-17 β -oestradiol, such as 2-methoxy-17 β -
5 oestradiol)
- 4-OH-17 β -oestradiol
- 6 α -OH-17 β -oestradiol
- 7 α -OH-17 β -oestradiol
- 2-OH-17 α -oestradiol
- 10 2-alkoxy-17 α -oestradiol (such as C₁₋₆ alkoxy-17 α -oestradiol, such as 2-methoxy-17 α -
oestradiol)
- 4-OH-17 α -oestradiol
- 6 α -OH-17 α -oestradiol
- 7 α -OH-17 α -oestradiol
- 15 16 α -OH-17 α -oestradiol
- 16 α -OH-17 β -oestradiol
- 16 β -OH-17 α -oestradiol
- 16 β -OH-17 β -oestradiol
- 17 α -oestradiol
- 20 17 β -oestradiol
- 17 α -ethinyl-17 β -oestradiol
- 17 β -ethinyl-17 α -oestradiol

oestriols and substituted oestriols, viz:

- 25 oestriol
- 2-OH-oestriol
- 2-alkoxy-oestriol (such as C₁₋₆ alkoxy-oestriol, such as 2-methoxy-oestriol)
- 4-OH-oestriol
- 6 α -OH-oestriol
- 30 7 α -OH-oestriol

dehydroepiandrosterones and substituted dehydroepiandrosterones, viz:

dehydroepiandrosterones

6 α -OH-dehydroepiandrosterone

7 α -OH-dehydroepiandrosterone

5 16 α -OH-dehydroepiandrosterone

16 β -OH-dehydroepiandrosterone

In general terms the ring system A'B'C'D' may contain a variety of non-interfering substituents. In particular, the ring system A'B'C'D' may contain one or more hydroxy, alkyl especially lower (C₁-C₆) alkyl, e.g. methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, n-pentyl and other pentyl isomers, and n-hexyl and other hexyl isomers, alkoxy especially lower (C₁-C₆) alkoxy, e.g. methoxy, ethoxy, propoxy etc., alkynyl, e.g. ethynyl, or halogen, e.g. fluoro substituents.

15 In an alternative embodiment, the polycyclic compound may not contain or be based on a steroid nucleus. In this regard, the polycyclic compound may contain or be based on a non-steroidal ring system - such as diethylstilboestrol, stilboestrol, coumarins, and other ring systems. Other suitable non-steroidal compounds for use in or as the composition of the present invention may be found in US-A-5567831.

20

In formula (I), the at least one sulphamate group is attached to any one or more of the ring components.

25 Preferably, the polycyclic compound has a steroidal structure and wherein the sulphamate group is attached to the A ring.

Preferably, the sulphamate group is attached to the 3 position of the A ring.

Preferably the sulphamate compound comprises at least one oxyhydrocarbyl group.

30

A preferred sulphamate compound is an oxyhydrocarbyl steroidal sulphamate compound (i.e. a sulphamate compound comprising a steroidal component and an oxyhydrocarbyl group).

5 In one embodiment, preferably, the sulphamate compound is an oxyhydrocarbyl steroidal sulphamate compound wherein the sulphamate group is in the 3 position on the steroidal component and/or the oxyhydrocarbyl group is in the 2-position position on the steroidal component.

10 In one embodiment, preferably, the sulphamate compound is an oxyhydrocarbyl derivative of oestrone sulphamate.

In one embodiment, preferably, the sulphamate compound is an oxyhydrocarbyl derivative of oestrone-3-*O*-sulphamate.

15

In one embodiment, preferably, the sulphamate compound is a C₁₋₆ (such as a C₁₋₃) alkoxy derivative of oestrone-3-*O*-sulphamate.

In one embodiment, preferably, the sulphamate compound is a 2-C₁₋₆ (such as a C₁₋₃)
20 alkoxy derivative of oestrone-3-*O*-sulphamate.

In one embodiment, preferably, the sulphamate compound is 2-methoxyoestrone-3-*O*-sulphamate.

25 The term "oxyhydrocarbyl group" as used herein means a group comprising at least C, H and O and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, an alkyl group, a cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of substituents may form a cyclic group. If the oxyhydrocarbyl group comprises more
30 than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group.

Thus, the oxyhydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in the art and include, for instance, sulphur and nitrogen.

5 In one preferred embodiment of the present invention, the oxyhydrocarbyl group is a oxyhydrocarbon group.

Here the term "oxyhydrocarbon" means any one of an alkoxy group, an oxyalkenyl group, an oxyalkynyl group, which groups may be linear, branched or cyclic, or an oxyaryl group. The term oxyhydrocarbon also includes those groups but wherein they
10 have been optionally substituted. If the oxyhydrocarbon is a branched structure having substituent(s) thereon, then the substitution may be on either the hydrocarbon backbone or on the branch; alternatively the substitutions may be on the hydrocarbon backbone and on the branch.

15 Preferably the oxyhydrocarbyl group is of the formula $C_{1-6}O$ (such as a $C_{1-3}O$).

If the sulphamate compound comprises a steroidal nucleus, preferably the A ring has an oxyhydrocarbyl group at the 2 position.

20 More preferably the group $C_{1-6}O$ is attached to the 2 position of the A ring of a steroidal nucleus.

Preferably, the oxyhydrocarbyl group is an alkoxy.

25 The alkyl group of the alkoxy substituent is preferably a lower alkyl group containing from 1 to 5 carbon atoms, that is to say methyl, ethyl, propyl etc. Preferably, the alkyl group is methyl.

Thus, in a preferred embodiment, if the sulphamate compound comprises a steroidal
30 nucleus the A ring has an methoxy substituent at the 2 position.

42

Preferably the sulphamate compound is suitable for use as an inhibitor of oestrone sulphatase (E.C. 3.1.6.2).

In one preferred embodiment of the present invention, preferably the sulphamate compound is non-oestrogenic. The term "non-oestrogenic" means exhibiting no or substantially no oestrogenic activity.

In one preferred embodiment of the present invention, preferably the sulphamate compound are not capable of being metabolised to compounds which display or induce hormonal activity.

In one preferred embodiment of the present invention, preferably the composition of the present invention is orally active.

The present invention is based on the highly surprising finding that the combination of a sulphamate compound and a biological response modifier provides an effective treatment of cancer.

More in particular, we have surprisingly found that the composition of the present invention - and 2-methoxyoestrone-3-*O*-sulphamate - can prevent or suppress glucose uptake by a tumour and/or prevent and/or inhibit tumour angiogenesis and/or disrupt microtubules and/or induce apoptosis.

In this respect, microtubules, together with microfilaments and intermediate filaments form part of the cytoskeletal system of a cell. Microtubules are responsible for many of cell movements-examples include the beating of cilia and flagella and the transport of membrane vesicles in the cytoplasm. All these movements result from the polymerisation and depolymerisation of microtubules or the actions of the microtubule motor proteins dynein and kinesins. Some other cell movements, such as the alignment and separation of chromosomes during meiosis and mitosis result from both mechanisms. Microtubules also direct cell movement but in some cases, microtubules serve purely structural functions.

A microtubule is composed of subunits that are heterodimers of α -tubulin and β -tubulin monomers. There are two populations of microtubules: stable, long-lived microtubules and dynamic, short lived microtubules. Dynamic microtubules are found when the microtubule structures need to assemble and disassemble quickly. For example, during mitosis, the cytosolic microtubule network characteristic of interphase cells disappears and the tubulin from it is used to form the spindle apparatus which partitions chromosomes equally to the daughter cells. When mitosis is complete, the spindle disassembles and the interphase microtubule network reforms.

Drugs that inhibit mitosis provide a useful means to manipulate the microtubules in a cell. Three drugs: colchicine, vinblastine and taxol - all purified from plants - have proved to be very powerful probes of microtubule function partly because they bind only to tubulin or microtubules and not to other proteins and also because their concentrations in cells can be easily controlled.

Because of their effects on mitosis, microtubule inhibitors have been widely used to treat illness and more recently as anticancer agents, since blockage of spindle formation will preferentially inhibit rapidly dividing cells like cancer cells. A highly effective anti-ovarian cancer agent is taxol. In ovarian cancer cells, which undergo rapid cell divisions, mitosis is blocked by taxol treatment while other functions carried out by intact microtubules are not affected. A comprehensive review of microtubules can be found in "Molecular Cell Biology" (Ed: Lodish et al 1995 WH Freeman and Co. New York pp 1051-1122).

Apoptosis is induced by MT-targeting drugs, a process which may involve the phosphorylation (and inactivation) of the apoptosis regulator, the bcl-2 protein (Halder, Cancer Res. 57: 229, 1997).

Preferably the composition of the present invention further comprises a pharmaceutically acceptable carrier, diluent, or excipient.

For pharmaceutical administration, the composition of the present invention can be formulated in any suitable manner utilising conventional pharmaceutical formulating techniques and pharmaceutical carriers, adjuvants, excipients, diluents etc. - such as those for parenteral administration. Approximate effective dose rates are in the range 100 to 800 mg/day depending on the individual activities of the compounds in question and for a patient of average (70Kg) bodyweight. More usual dosage rates for the preferred and more active compositions will be in the range 200 to 800 mg/day, more preferably, 200 to 500 mg/day, most preferably from 200 to 250 mg/day. They may be given in single dose regimes, split dose regimes and/or in multiple dose regimes lasting over several days. For oral administration they may be formulated in tablets, capsules, solution or suspension containing from 100 to 500 mg of composition per unit dose. Alternatively and preferably the compositions will be formulated for parenteral administration in a suitable parenterally administrable carrier and providing single daily dosage rates in the range 200 to 800 mg, preferably 200 to 500, more preferably 200 to 250 mg. Such effective daily doses will, however, vary depending on inherent activity of the active ingredient and on the bodyweight of the patient, such variations being within the skill and judgement of the physician.

The composition or compound of the present invention may be administered in any suitable manner - such as any one or more of oral administration, topical administration (such as by means of a patch), parenteral administration, rectal administration or by inhalation spray.

In the method of treatment, the subject is preferably a mammal, more preferably a human. For some applications, preferably the human is a woman.

For particular applications, it is envisaged that the compositions of the present invention may be used in combination therapies, either with another sulphatase inhibitor, or, for example, in combination with an aromatase inhibitor, such as for example, 4-hydroxyandrostenedione (4-OHA).

In accordance with the present invention, the components of the composition can be added in admixture, simultaneously or sequentially. Furthermore, in accordance with the present invention it may be possible to form at least a part of the composition *in situ* (such as *in vivo*) by inducing the expression of - or increasing the expression of - one of the components. For example, it may be possible to induce the expression of - or increase the expression of - the biological response modifier, such as TNF. By way of example, it may be possible to induce the expression of - or increase the expression of - TNF by adding bacterial lipopolysaccharide (LPS) and muramyl dipeptide (MDP). In this regard, bacterial LPS and MDP in combination can stimulate TNF production from murine spleen cells *in vitro* and tumour regression *in vivo* (Fuks *et al* Biull Eksp Biol Med 1987 104: 497-499).

In addition, the present invention contemplates the composition of the present invention further comprising an inducer of the biological response modifier - such as *in vivo* inducer of the biological response modifier.

The present invention also contemplates the combination of an oxyhydrocarbonyl steroidal sulphamate compound according to the present invention (such as 2-methoxyoestrone-3-O-sulphamate) with an inducer of a biological response modifier - such as an *in vivo* inducer of an *in situ* biological response modifier.

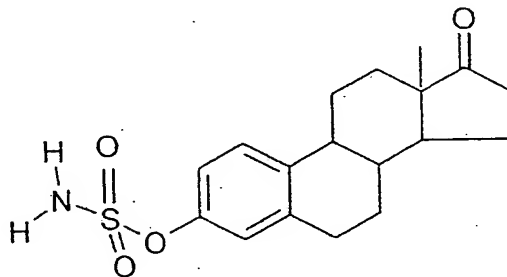
Examples of suitable sulphamate compounds for use in or as the composition of the present invention, or examples of suitable compounds that can be converted to suitable sulphamate compounds for use in or as the composition of the present invention, can be found in the art - such as PCT/GB92/01587, PCT/GB97/03352, PCT/GB97/00444, GB 9725749.7, GB 9725750.5, US-A-5567831, US-A-5677292, US-A-5567831, WO-A-96/05216, and WO-A-96/05217.

By way of example, PCT/GB92/01587 teaches novel steroid sulphatase inhibitors and pharmaceutical compositions containing them for use in the treatment of oestrone dependent tumours, especially breast cancer. These steroid sulphatase inhibitors are

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5 sulphamate esters. Examples of such inhibitors are sulphamate ester derivatives of steroids.

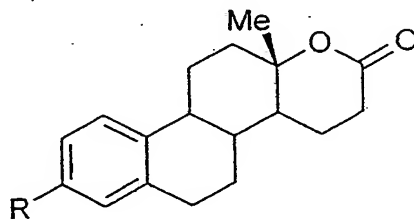
A compound suitable for use in the present invention - which is also a preferred compound of PCT/GB92/01587 - is oestrone-3-sulphamate (otherwise known as "EMATE"), which has the following structure:



10 It is known that EMATE is a potent E1-STS inhibitor as it displays more than 99% inhibition of E1-STS activity in intact MCF-7 cells at 0.1 μ M. EMATE also inhibits the E1-STS enzyme in a time- and concentration-dependent manner, indicating that it acts as an active site-directed inactivator.

15 Preferably, the A ring has a substituent that is an oxyhydrocarbyl group.

Another compound suitable for use in the present invention has at least the following skeletal structure:

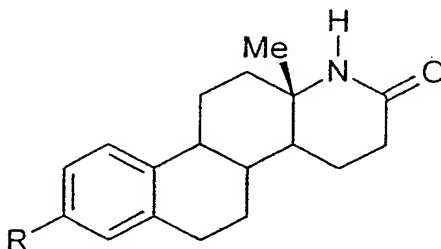


20 wherein R denotes a sulphamate group as described above.

Preferably, R is the above-mentioned preferred formula for the sulphamate group. In this regard, it is preferred that at least one of R_1 and R_2 is H.

Preferably, the A ring has a substituent that is an oxyhydrocarbyl group.

Another compound suitable for use in the present invention has at least the following skeletal structure:



wherein R denotes a sulphamate group as described above.

Preferably, R is the above-mentioned preferred formula for the sulphamate group. In this regard, it is preferred that at least one of R_1 and R_2 is H.

Preferably, the A ring has a substituent that is an oxyhydrocarbyl group.

In accordance with a preferred aspect of the present invention, if the sulphamate group of the compound were to be replaced with a sulphate group to form a sulphate compound then that sulphate compound would be hydrolysable by an enzyme having steroid sulphatase (E.C. 3.1.6.2) activity - i.e. when incubated with steroid sulphatase EC 3.1.6.2 at pH 7.4 and 37°C.

In one preferred embodiment, if the sulphamate group of the compound were to be replaced with a sulphate group to form a sulphate compound then that sulphate compound would be hydrolysable by an enzyme having steroid sulphatase (E.C. 3.1.6.2) activity and would yield a K_m value of less than 50mMoles when incubated with steroid sulphatase EC 3.1.6.2 at pH 7.4 and 37°C.

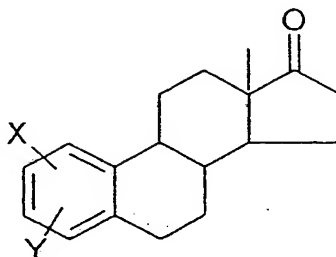
In another preferred embodiment, if the sulphamate group of the compound were to be replaced with a sulphate group to form a sulphate compound then that sulphate compound would be hydrolysable by an enzyme having steroid sulphotase (E.C. 3.1.6.2) activity and would yield a K_m value of less than 50 μ Moles when incubated with steroid sulphotase EC 3.1.6.2 at pH 7.4 and 37°C.

In a further aspect the present invention provides use of a sulphamate compound for the manufacture of a medicament to prevent and/or inhibit tumour growth; wherein the sulphamate compound is suitable for use as an inhibitor of oestrone sulphotase (E.C. 3.1.6.2); wherein the compound is a polycyclic compound having a steroidal structure, or a bio-isostere thereof; wherein the polycyclic compound comprises at least one sulphamate group attached to the A ring; and wherein the polycyclic compound comprises at least one oxyhydrocarbyl group attached to the A ring.

We have found that sulphamate compounds having an oxyhydrocarbyl substituent on the A ring are potent (and in some cases highly potent) in (i) preventing and/or inhibiting glucose uptake of a tumour and/or (ii) preventing and/or inhibiting tumour angiogenesis and/or (iii) disrupting microtubules and/or iv) inducing apoptosis.

Thus in a further aspect the present invention provides use of a sulphamate compound for the manufacture of a medicament to prevent and/or inhibit glucose uptake of a tumour and/or to prevent and/or inhibit tumour angiogenesis and/or to disrupt microtubules and/or induce apoptosis; wherein the sulphamate compound is suitable for use as an inhibitor of oestrone sulphotase (E.C. 3.1.6.2); wherein the compound is a polycyclic compound having a steroidal structure or a bio-isostere thereof; wherein the polycyclic compound comprises at least one sulphamate group attached to the A ring; and wherein the polycyclic compound comprises at least one oxyhydrocarbyl group attached to the A ring.

A preferred sulphamate compound of the present invention has the formula:



5 wherein X is an oxyhydrocarbyl group; and Y is a sulphamate group; and

wherein rings A, B, C and D are independently optionally substituted.

Preferably Y is in the 3-position.

10

Preferably X is in the 2-position.

15

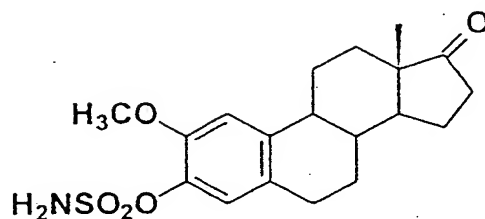
For the present invention, preferably the sulphamate compound is an oxyhydrocarbyl steroidal sulphamate compound, in particular 2-methoxyoestrone-3-*O*-sulphamate, or a pharmaceutically active salt thereof, including analogues thereof.

2-methoxyoestrone-3-*O*-sulphamate is an analogue of EMATE - and can be called 2-methoxy EMATE.

20

2-methoxy EMATE is the sulphamoylated derivative of a naturally occurring oestrogen metabolite, 2-methoxyoestrone. This compound is formed in the liver by the hydroxylation of oestrone by a 2-hydroxylase, with subsequent metabolism to the methoxy derivative by catechol oestrogen methyl transferase.

2-methoxy EMATE has the formula presented as formula below:



2-methoxy EMATE is believed to act *in vivo*, at least in part, by inhibiting tumour
5 angiogenesis.

Thus, in a highly preferred embodiment the sulphamate compound is an oxyhydrocarbyl
steroidal sulphamate compound, in particular 2-methoxyestrone-3-*O*-sulphamate (2-
methoxy EMATE).

10

In this regard, we have found that a sulphamate compound having a C₁₋₆ (such as a C₁₋₃)
alkoxy substituent at the 2 position of the A ring, in particular 2-methoxy EMATE, is
highly potent in preventing and/or inhibiting growth of tumours.

15 The present invention also provides compositions/compounds which:

cause inhibition of growth of oestrogen receptor positive (ER+) and ER negative
(ER-) breast cancer cells *in vitro* by induction of apoptosis.

20 cause regression of nitroso-methyl urea (NMU)-induced mammary tumours in
intact animals (i.e. not ovariectomised).

inhibit the uptake of glucose in cancer cells, in particular in breast cancer cells
and breast tumour-derived fibroblasts.

25

induce apoptosis.

disrupt microtubules (Mts).

act *in vivo* by inhibiting angiogenesis.

5 The sulphamate compounds of the present invention may be prepared by reacting an appropriate alcohol with the appropriate sulfamoyl chloride, $R_1R_2NSO_2Cl$. Preferred conditions for carrying out the reaction are as follows. Sodium hydride and a sulfamoyl chloride are added to a stirred solution of the alcohol in anhydrous dimethyl formamide at 0°C. Subsequently, the reaction is allowed to warm to room temperature whereupon
10 stirring is continued for a further 24 hours. The reaction mixture is poured onto a cold saturated solution of sodium bicarbonate and the resulting aqueous phase is extracted with dichloromethane. The combined organic extracts are dried over anhydrous $MgSO_4$. Filtration followed by solvent evaporation *in vacuo* and co-evaporated with toluene affords a crude residue which is further purified by flash chromatography. Preferably, the alcohol
15 is derivatised, as appropriate, prior to reaction with the sulfamoyl chloride. Where necessary, functional groups in the alcohol may be protected in known manner and the protecting group or groups removed at the end of the reaction.

In summation, the present invention provides compositions for use in treatment of tumours
20 and pharmaceutical compositions containing them.

The present invention will now be described only by way of example, in which reference, in which reference shall be made to the following Figures.

25 Figure 1 which is a photographic plate (Plate 1);
Figure 2 which is a photographic plate (Plate 2);
Figure 3 which is a photographic plate (Plate 3);
Figure 4 which is a photographic plate (Plate 4);
Figure 5 which is a photographic plate (Plate 5);
30 Figure 6 which is a photographic plate (Plate 6);
Figure 7 which is a photographic plate (Plate 7);
Figure 8 which is a photographic plate (Plate 8);

Figure 9 which is a bar chart;

Figure 10 which is a bar chart;

Figure 11 which is graph; and

Figure 12 which is graph.

5

Synthesis of 2-methoxyoestrone-3-*O*-sulphamate (2-methoxy EMATE)

2-methoxy EMATE was synthesised by treating a solution of 2 methoxyoestrone in anhydrous dimethylformamide with sodium hydride at 0°C. After evolution of hydrogen had ceased sulphamoyl chloride (2 equiv.) was added and the reaction mixture was allowed to warm to room temperature overnight. The compound was purified by silica gel flash chromatography, was a single pure spot by TLC and exhibited satisfactory spectroscopic and microanalytical data.

In this regard, 2-Methoxy oestrone (75 mg, 0.250 mmol) gave a crude product (103 mg) which was fractionated on silica (50 g) with chloroform/acetone (8:1) and upon evaporation the second fraction gave a pale white residue (83 mg, 81%) which was recrystallized in ethylacetate/hexane (1:2) to give 1 as white crystals (69 mg) .m.p = 177-180°C, R_f = 0.29 and 0.54 for chloroform/ acetone 8:1 and 4:1 respectively and 0.46 and 0.31 for ethylacetate/hexane 2:1 and 1:1 respectively. ν_{\max} (KBr) 3400, 3300 (-NH₂), 1610 (C=O), and 1380 (-SO₂N-) cm⁻¹. δ_H (CDCl₃) 0.922 (3H, s, C-18-CH₃), 1.24- 2.87 (15H, m), 3.88 (3H, s, C-2-OCH₃), 5.0 (2H, br s, exchanged with D₂O, -SO₂NH₂), 6.93 (1H, s, C-1- H) and 7.06 (1H, s, C-4-H). MS: m/z (+ve ion FAB in m-NBA, rel. intensity) 379.1 [100, (M)⁺], 300.0 [25, (M-SO₂NH₂)⁺]. MS: m/z (-ve ion FAB in m-NBA, rel. intensity) 378.0 [100, (M-H)]. Acc. MS: m/z (FAB⁺) = 380.1515 C₁₉H₂₆NO₅S requires 380.1532 Found C, 60.0; H, 6.7; N, 3.67; C₁₉H₂₅NO₅S requires C, 60.14; H, 6.64; N, 3.69%.

Experimental Studies

1. Effect of 2-methoxy EMATE on cell growth and apoptosis

- 5 Apoptotic cells undergo rounding, become detached from their neighbours and are easily detected by light microscopy. An estimate of the proportion of apoptotic cells was made by counting the number of rounded cells in ten microscopic fields. Representative photomicrographs of control and treated cells are shown.

10	Plate No.	% Apoptotic Cells
	Plate 1	
	MCF-7 (ER+) breast cancer cells. Controls.	< 1
15	Plate 2	
	MCF-7 cells + 2-methoxy EMATE (5 μ M, 72h)	> 90
	Plate 3	
20	MDA-MB231 (ER-) breast cancer cells. Controls.	< 2
	Plate 4	
	MDA-MB-231 + 2-methoxy EMATE (1 μ M, 24h)	> 50
25	Plate 5	
	Breast tissue-derived fibroblasts (BTFs). Controls.	< 1
	Plate 6	
30	BTFs + 2-methoxy EMATE (1 μ M, 24h)	> 30

2. Effect of 2-methoxy EMATE on tumour growth *in vivo*

- To examine the ability of 2-methoxy EMATE to inhibit tumour growth in animals,
 35 Ludwig rats were obtained from Harlan-Olac (UK) Ltd after induction of mammary tumours with nitroso-methyl urea (NMU). For tumour induction 50-day-old rats were injected *via* the tail vein with NMU (50 mg/kg) receiving 3 injections at 14-day

intervals. Tumour development was monitored and upon reaching 1.0 - 1.5 cm in diameter animals were given 2-methoxy EMATE (20 mg/kg/d for 11 days) or vehicle (propylene glycol).

- 5 Tumours (Vol D_{11}) in animals receiving vehicle only for 11 days remained static or increased by 109% compared with tumour volumes at the start of the treatment period (Vol D_0) (Table I). In contrast in 2/3 animals treated with 2-methoxy EMATE tumours showed complete regression within the 11-day treatment period. These are intact animals and therefore still produce oestrogen from their ovaries, but 2 months later no
10 recurrence of tumour growth has been detected. The tumour in the 3rd animal was larger and may have required a higher dose/longer period of treatment to cause regression.

15 **Table I** Effect of 2-methoxy EMATE on *in vivo* tumour growth

<u>Controls (Vol D_{11} / Vol D_0 %)</u>		<u>2-methoxy EMATE (Vol D_{11} / Vol D_0 %)</u>
1	100	< 10
2	155	< 13
3	209	144 (no response)

25 3. Effect of 2-methoxy EMATE on glucose uptake

MCF-7 cells or fibroblasts were seeded into 24- well tissue culture plates and grown until approximately 80% confluent. Cells were washed twice with phosphate-buffered
30 saline (PBS, 5 ml). 2-methoxy EMATE was added in glucose-free RPMI culture medium (1 ml) containing 2-deoxyglucose (1 μ Ci, Amersham International). Cells were incubated for 15 min at 37°C after which they were washed twice with cold (0-4°C) PBS (5 ml). Cells were solubilized using 0.2% Triton X-100 in 0.01M NaOH (1 ml). Cell

Table II Inhibition of Glucose Uptake

	<u>% Controls</u>
MCF-7 cells + 2-methoxy EMATE (10 μ M)	51
Breast tumour-derived fibroblasts + 2-methoxy EMATE (10 μ M)	36

15 At 10 μ M 2-methoxy EMATE resulted in a significant (49% and 74% respectively) inhibition of glucose uptake by MCF-7 cells and fibroblasts respectively.

4. Effect of 2-methoxy EMATE on Glucose uptake

20 For uptake assays cells were plated into 12-well multi-well plates and grown to confluence. Cells were washed with PBS and incubated for 15min in incubation buffer containing 1μCi 2-deoxy-D-[1-³H] glucose (26.2Ci/mmol, NEN-Dupont, UK) per well in the absence or presence of potential inhibitors (0.1-10μM). Uptake was terminated by washing the cells in cold (4⁰C) PBS. The cells were solubilized in Triton-x in 0.01M NaOH and processed
25 for liquid scintillation counting. Cell number was determined using parallel wells and counting as described below under Cell Culture & Counting.

Results

30 As apoptosis in transformed cells can be induced by glucose deprivation the ability of 2-MeOEMATE to inhibit glucose uptake was examined. Using MCF-7 cells uptake of deoxyglucose was shown to be linear with respect to cell number over the range 0.1-1.2 x

10^6 cells and over a 5-35 min period. Deoxyglucose uptake experiments were usually carried out with a 15min incubation period.

The ability of two known inhibitors of glucose uptake, cytochalasin B and the isoflavone genistein, to inhibit uptake using this model was initially examined (22). Cytochalasin B (10 μ M) inhibited deoxyglucose uptake by 91% while the effect of genistein, and its sulphamoylated derivative, at this concentration was lower (25%-42% inhibition) (Fig 11a). At 100 μ M genistein and its sulphamoylated derivative inhibited deoxyglucose uptake by 82% and 79% respectively (data not shown). An examination of the ability of two estrogen conjugates to inhibit uptake revealed that oestrone-3-sulphate was without effect whereas oestrone-3-glucuronide inhibited uptake by 29% (Fig 11a).

The effect of a number of estrogen metabolites on deoxyglucose uptake by MCF-7 cells is shown in Fig 11b. 2-Hydroxyoestradiol, 2-MeOE2 or 2-MeOE1S had little effect on glucose uptake. In contrast, 2-MeOE1 and 2-MeOEMATE inhibited deoxyglucose uptake in a dose-dependent manner with 42% and 49% inhibition respectively occurring at 10 μ M. While the extent of inhibition of deoxyglucose uptake resulting from exposure of cells to 2-MeOEMATE is similar to that observed for genistein, it is lower than that resulting from treatment of cells with cytochalasin B. 2-MeOEMATE (10 μ M) also inhibited uptake of deoxyglucose in breast tumour-derived fibroblasts by 64% (data not shown).

Cell Culture & Counting

MCF-7 (estrogen receptor+ [ER+]) and MDA-MB-231 (ER-) breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in 25cm² culture flasks in Eagle's minimum essential medium (EMEM) with Hepes buffer (20mM). This medium was supplemented with L-glutamine (2mM), sodium hydrogen carbonate (10mM), 1% non-essential amino acids and 5% (v/v) foetal calf serum (FCS). Before adding test compounds, cells were washed with phosphate-buffered saline (PBS) and treatments added in phenol-red free medium containing 2% stripped FCS and supplements. The effects of 2-MeOE1 or 2-MeOEMATE on the growth of MCF-7 cells was assessed using a Cell Titer 96 cell proliferation assay (Promega,

Southampton, Hants. UK) according to the manufacturers' instructions. For this, cells (5000 per well) were cultured in medium containing phenol-red and 10% FCS and were exposed to a drug for 4 days before the assay was performed. For MDA-MB-231 cells, cell numbers were determined using a Coulter counter.

5

For the culture of fibroblasts, resected breast tumour tissue was minced and incubated in EMEM for 18-24h at 37°C with collagenase (200µg/ml). The dispersed cells were harvested by centrifugation and washed twice with medium to remove collagenase. Dispersed cells were seeded into culture flasks and grown to confluence before passaging on a weekly basis. For experimental purposes 12 well multi-well plates or 25cm² flasks were seeded with fibroblasts and grown to 70-80% confluency. Cells were washed with PBS and exposed to drugs for 24h before determining cell numbers using a Coulter counter.

10

Photomicrographs of control and treated cells were taken under normal conditions of light and exposure using an Olympus SL35 Type 12 camera under an Olympus CK2 microscope (x100 magnification).

15

5. Tdt-mediated dUTP-nick end labelling (TUNEL) analysis

20

The ability of 2-MeOEMATE to induce apoptosis in MCF-7 cells was examined by TUNEL analysis using an *in situ* cell death detection kit (Boehringer Mannheim UK Ltd., Lewes, East Sussex, UK). Cells were fixed and permeabilised according to the manufacturers' instructions. Stained apoptotic cells were quantitated by flow cytometry.

25

Results

This possibility was confirmed in a further experiment by TUNEL analysis (Fig 8). For untreated cells no increase in the proportion of fluorescently labelled cells was detected after staining. In contrast, there was a significant increase in the proportion of fluorescently labelled cells after exposure to 2-MeOEMATE (10µM) for 48h.

30

Fluorescently labelled cells represented approximately 10% of the cell population. This result indicates that 2-MeOEMATE can induce cells to undergo apoptosis.

6. Effect of 2-MeOEMATE on growth of NMU-induced mammary tumours in intact rats

5

The effect of 2-MeOEMATE on mammary tumour growth was examined in a preliminary study using Ludwig rats (Harlan Olac, Bicester, UK) in which tumours were induced by inoculation of NMU. Tumour development was monitored regularly and when 0.5-1.0cm³ in volume, animals received vehicle (propylene glycol, 200µl/day, p.o.), 2-MeOEMATE (20mg/kg/day, p.o.) or 2-MeOE1 (20mg/kg/day, p.o.) daily for an 11 day period. Tumour length and width was measured with callipers and tumour volumes calculated as described (21).

Results

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A preliminary study was carried out to compare the abilities of 2-MeOE1 and 2-MeOEMATE to inhibit tumour growth *in vivo*. For this, the growth of mammary tumours was initiated by inoculation with NMU. Drugs were administered orally when tumour volumes reached 0.5-1.0cm³. For two of the animals receiving vehicle, tumour volumes continued to increase (average 82%) while little change in the volume of a tumour in a third animal was detected (Fig 12). For two animals receiving 2-MeOE1 no change in tumour volume occurred in one, while for the other a modest (25%) reduction was detected over the 11-day period of the study.

For three animals receiving 2-MeOEMATE the tumour volume in one animal continued to increase up to day 6, but thereafter showed a slight (8%) reduction. In contrast, for the two other animals receiving 2-MeOEMATE, tumours regressed rapidly and were barely palpable at the end of the 11-day period. Tumour volumes in the two animals receiving 2-MeOEMATE that regressed were monitored for a further 33 days during which time no regrowth of tumours was detected.

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Hormono-ImmunoTherapy (H.I.T.)

Tumour necrosis factor α (TNF α) is a cytokine produced by macrophages, lymphocytes and other cells in the body. Recombinant TNF α has been used to treat a number of different types of cancer but so far has only met with limited success. In humans the severe side-effects induced by this cytokine have restricted its use for cancer therapy.

Our studies have revealed that a combination of an oxyhydrocarbyl steroidal sulphamate compound (in particular 2-methoxy EMATE/EMATE) with a biological response modifier (in particular TNF α) may enhance the efficacy of this form of therapy.

A *In vitro* study

Plates 1 and 2 have previously illustrated the effect of 2-methoxy EMATE (5 μ M) on apoptosis induction in MCF-7 breast cancer cells (for details on these cells see PCT/GB92/01587). This study was extended to examine:

TABLE III

<u>Plate No.</u>	<u>% Apoptotic cells</u>
Plate 7 MCF-7 cells + TNF α (10 ng/ml)	< 1
Plate 8 MCF-7 cells + 2-methoxy EMATE (1 μ M) + TNF α (10 ng/ml)	> 90

In this experiment significantly less cells were present than in cells treated with only 2-methoxy EMATE. This finding was confirmed by counting the number of cells as shown in Figure 9.

To assess the significance of this *in vitro* observation, an intact rat with an NMU-induced mammary tumour was treated with EMATE (20 mg/kg p.o.) for 3 days. It is

generally known that oestrogens are metabolised *via* 2-hydroxylation with subsequent methylation. For days 2 and 3 in addition to EMATE, rat rTNF α (5 μ g) was administered i.p. The dose appeared to be well tolerated.

- 5 By day 3, the tumour volume had decreased by 72% compared with its volume before the start of treatment (Figure 10). However, 6 days after the cessation of EMATE/TNF α therapy, the tumour had increased in volume by 120% compared with its Day 0 volume.
- 10 These results indicate that a combination of an oxyhydrocarbyl steroidal sulphamate compound and a steroidal sulphamate compound (in particular 2-methoxy EMATE/EMATE) plus a biological response modifier (in particular TNF α) may offer considerable therapeutic advantage for the treatment of tumours.

15 2 Methoxyoestrone

In this comparative example, two intact rats with NMU-induced mammary tumours were treated with 2 methoxyoestrone (20mg/kg/d, p.o.) for 11 days. Tumour volumes were determined before (Day 0 volume) and at the end of the treatment with 2

20 methoxyoestrone (Day 11 volume).

The results are shown below:

25 **Table IV - Effect of 2 methoxyoestrone on *in vivo* tumour growth**

	<u>Vol D₁₁/Vol D₀ %</u>
1	75
2	100

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These data highlight the surprising nature of the present invention.

DISCUSSION

Our findings show that it is possible to treat cancer with a combination of a sulphamate
5 compound and a biological response modifier.

Our findings show that the combination of a sulphamate compound and a biological
response modifier acts to inhibit cell-tumour growth by reducing glucose uptake by
cancer cells and tumour-derived fibroblasts.

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Our findings also indicate that the oxyhydrocarbyl steroidal sulphamate compound
according to the present invention, especially 2-methoxy EMATE, acts to inhibit cell-
tumour growth by reducing glucose uptake by cancer cells and tumour-derived
fibroblasts. It is known that many cancers have an increased uptake of glucose and an
15 increased rate of glucose metabolism. Transformation of cell lines results in the
elevation of a protein that is involved in glucose uptake (glucose transporter, Glut 1).
3T3 Fibroblasts transfected with *ras/src* have an increased uptake of glucose (Flier *et al.*,
Science 235: 1492, 1987). Whereas glucose deprivation of normal rat fibroblasts
did not induce apoptosis, glucose deprivation of c-myc transfected fibroblasts resulted in
20 extensive apoptosis (Shim *et al.*, *Proc Natl Acad Sci*, *USE* 95: 1511, 1998). Glut 1 is
over expressed in breast tumours (Brown and Wahl, *Cancer* 72: 2979, 1993) but is not
detectable in normal or benign breast tissues (Younes *et al.*, *Cancer Res.* 56: 1164,
1996). Since cancer cells do not accumulate an intracellular store of glucose in the form
of glycogen or fat, it must be obtained continuously from an external source and
25 transported into the cell.

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can also disrupt microtubules.

30 A key advantage of the present invention is that the oxyhydrocarbyl steroidal sulphamate
compound according to the present invention, especially 2-methoxy EMATE, can also
disrupt microtubules.

A key advantage of the present invention is that the composition of the present invention can induce apoptosis.

5 Another key advantage of the present invention is that the oxyhydrocarbyl steroidal sulphamate compound according to the present invention, especially 2-methoxy EMATE, can induce apoptosis. In this regard, while previous investigations have suggested that 2-methoxy E2 has potent anti-mitotic properties, results from our studies indicate that 2-methoxy EMATE inhibits cell growth by inducing apoptosis. Tumours grow if either
10 the rate of cell growth (proliferation) is increased or rate of cell death (apoptosis) is decreased. In apoptosis, cells round up, sever connection with their neighbouring cells and DNA cleaves into oligonucleosomal fragments.

A further key advantage of the present invention is that the oxyhydrocarbyl steroidal
15 sulphamate compound according to the present invention, especially 2-methoxy EMATE, can prevent and/or inhibit tumour angiogenesis.

A further key advantage of the present invention is that the composition of the present invention can prevent and/or inhibit tumour angiogenesis.

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The present invention also provides compositions/compounds which:

- 1 cause inhibition of growth of oestrogen receptor positive (ER+) and ER negative (ER-) breast cancer cells *in vitro* by induction of apoptosis.
- 25 2 cause complete regression of $2/3$ rd of nitroso-methyl urea (NMU)-induced mammary tumours in intact (i.e. not ovariectomised) rats in 11 days.
- 3 inhibited the uptake of glucose in cancer cells, in particular in breast cancer cells
30 and breast tumour-derived fibroblasts.

4 induce apoptosis, it is believed by disrupting microtubules (MTs), which form part of the cytoskeleton.

5. act *in vivo* by inhibiting angiogenesis.

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Thus, in summary, the present invention provides a composition and compound suitable for use in the treatment of cancers and, especially, breast cancer.

10 In particular, in one aspect the present invention addresses the problem of blocking the growth of tumours in endocrine-dependent tissues (e.g. breast, endometrium, prostate). Nevertheless, other tumours (e.g. sarcomas, melanomas) should also be amenable to treatment with the composition and compound of the present invention.

15 It is also believed that the present invention has implications in treating hormonal conditions in addition to those associated with oestrogen. Hence, the present invention also provides a composition that is capable of affecting hormonal activity and is capable of affecting an immune response, wherein the composition is the composition of the present invention.

20 It is also to be understood that the composition of the present invention may have other important medical implications.

For example, the composition of the present invention may be useful in the treatment of the disorders listed in WO-A-98/05635. For ease of reference, part of that list is now
25 provided: cancer, inflammation or inflammatory disease, dermatological disorders, fever, cardiovascular effects, haemorrhage, coagulation and acute phase response, cachexia, anorexia, acute infection, HIV infection, shock states, graft-versus-host reactions, autoimmune disease, reperfusion injury, meningitis, migraine and aspirin-dependent anti-thrombosis; tumour growth, invasion and spread, angiogenesis,
30 metastases, malignant, ascites and malignant pleural effusion; cerebral ischaemia, ischaemic heart disease, osteoarthritis, rheumatoid arthritis, osteoporosis, asthma, multiple sclerosis, neurodegeneration, Alzheimer's disease, atherosclerosis, stroke,

vasculitis, Crohn's disease and ulcerative colitis; periodontitis, gingivitis; psoriasis, atopic dermatitis, chronic ulcers, epidermolysis bullosa; corneal ulceration, retinopathy and surgical wound healing; rhinitis, allergic conjunctivitis, eczema, anaphylaxis; restenosis, congestive heart failure, endometriosis, atherosclerosis or endosclerosis.

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In addition, or in the alternative, the composition of the present invention may be useful in the treatment of disorders listed in WO-A-98/07859. For ease of reference, part of that list is now provided: cytokine and cell proliferation/differentiation activity; immunosuppressant or immunostimulant activity (e.g. for treating immune deficiency, including infection with human immune deficiency virus; regulation of lymphocyte growth; treating cancer and many autoimmune diseases, and to prevent transplant rejection or induce tumour immunity); regulation of haematopoiesis, e.g. treatment of myeloid or lymphoid diseases; promoting growth of bone, cartilage, tendon, ligament and nerve tissue, e.g. for healing wounds, treatment of burns, ulcers and periodontal disease and neurodegeneration; inhibition or activation of follicle-stimulating hormone (modulation of fertility); chemotactic/chemokinetic activity (e.g. for mobilising specific cell types to sites of injury or infection); haemostatic and thrombolytic activity (e.g. for treating haemophilia and stroke); antiinflammatory activity (for treating e.g. septic shock or Crohn's disease); as antimicrobials; modulators of e.g. metabolism or behaviour; as analgesics; treating specific deficiency disorders; in treatment of e.g. psoriasis, in human or veterinary medicine.

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In addition, or in the alternative, the composition of the present invention may be useful in the treatment of disorders listed in WO-A-98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune response, including a response not associated with inflammation; inhibit the ability of macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reaction and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases,

inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididymo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases; posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fundus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillain-Barre syndrome, Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and

peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplantation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with
5 a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone
10 marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

All publications and patents mentioned in the above specification are herein incorporated by reference.

15 Various modifications and variations of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for
20 carrying out the invention which are obvious to those skilled in chemistry, biology or related fields are intended to be within the scope of the following claims.